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DEVELOPMENT AND EVALUATION OF NEW STAINS AND OTHER OPTICAL MARKERS USEFUL FOR CYTOPATHOLOGIC SPECIMENS IN SUSPENSION

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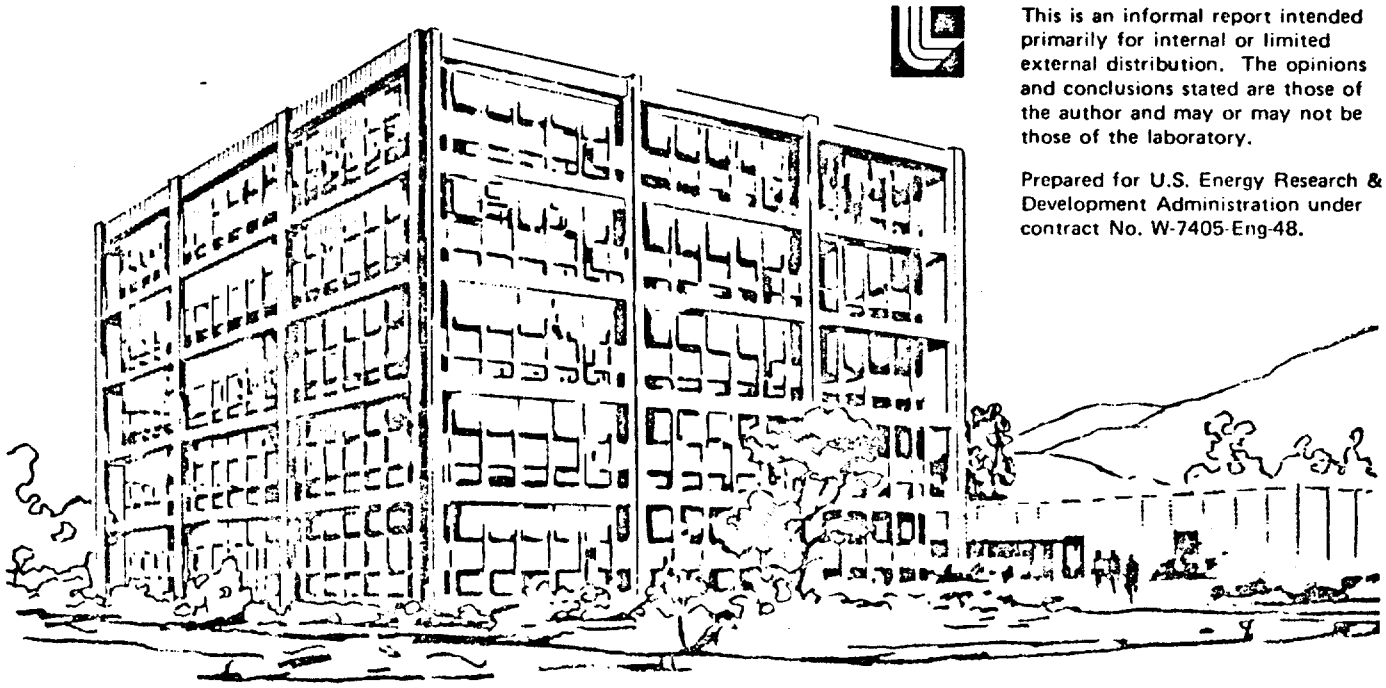
Biomedical Sciences Division

Tenth Semi-Annual Progress Report



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LAWRENCE LIVERMORE LABORATORY

TENTH SEMI-ANNUAL PROGRESS REPORT

DOE/NIH INTERAGENCY AGREEMENT Y-01-CB-40300

"Development and Evaluation of New Stains and Other Optical Markers useful for Cytopathologic Specimens in Suspension"

Period Covered:	1 December 1978 - 30 June 1979
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Ronald H. Jensen

Ronald H. Jensen, Ph.D.
(For B.H.M.)

TABLE OF CONTENTS

SUMMARY PROGRESS REPORT

The major part of our effort during this past reporting period has been engineering design, assembly, and testing of the Dual Beam, Computer Controlled Sorter. At the same time, double DNA staining and single DNA staining have continued to be studied.

Specific achievements during the reporting period are:

- Redesign of the signal handling electronics in the DBS.
- Assembly of the redesigned electronics and installation into the DBS.
- Completion of the firmware installation of the ND-6660 for computer-controlled sorting.
- Addition of a video display to the LSI-11 sorter monitor.
- Three parameter analysis of cervical samples measuring log (chromomycin A3 fluorescence), log (orthogonal light scatter), log (Hoechst 33258 fluorescence).
- Three parameter analysis of cervical samples measuring log (chromomycin A3 fluorescence), log (Orthogonal light scatter), log (SITS fluorescence).
- Analysis of the effect of DNase pretreatment on diagnostic power of flow cytometry measuring log (chromomycin A3 fluorescence) and log (orthogonal light scatter).
- Analysis of the diagnostic power of such flow cytometry on samples pooled separately from the ectocervix-endocervix and the vagina.

DETAILED PROGRESS REPORT

Dual Beam Sorter and Computer Controlled Sorting

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Workscope

1. Continue to implement and evaluate the dual laser sorter as described in Technical Proposal dated 1 March 1978. The Contractor shall determine "the basic operational specifications of the instrument" by testing each detector "in turn and then as part of the multiparameter system". Performance of the instrument will be evaluated on stain combinations used to evaluate gynecologic samples from normal and premalignant and malignant disease states which have been stained with two or more "fluorophores."

3. Develop and analyze computer controlled sorting for the dual-laser sorting system as described in the Technical Proposal dated 1 March 1978.

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Dual Beam Sorting

Major changes in the signal handling electronics have been implemented during this reporting period. The previous circuitry (Figure 1) encountered two important difficulties. 1) Thresholding of the signal processors was difficult to adjust and to maintain, thus the signal processors were often detecting signals due to debris or to background. 2) Direct transfer of signals from the amplifiers to the signal processors resulted in holding and measuring small signals due to cross-talk from laser beam one into the channels designated for laser beam two. This resulted in artifactual histograms in which the parameters from laser beam two were in error.

The present signal handling logic (Figure 2) is designed to eliminate these two problems. Threshold values of the signals from the amplifiers are checked by a discriminator (Disc.), which can be set more easily and precisely than a signal processor threshold. Signals from these discriminators are used to gate the signal processors (Sig Proc.) so as to allow acceptance of the valid signals. This improvement prevents the acceptance of many signals due to debris and background. The channels for laser beam two (channel 3 & 4 in the figure) are further processed by a gate and delay circuit attached to channel 1. The delay is set so as to accept signals

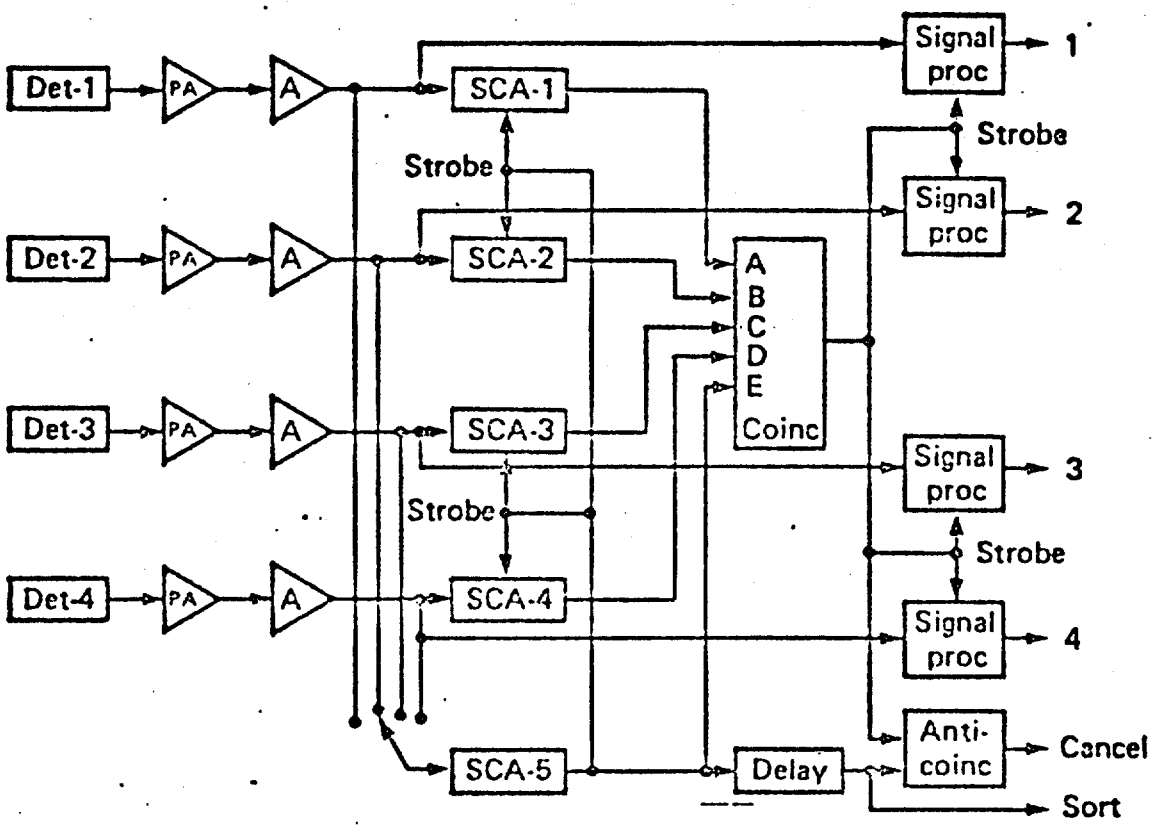


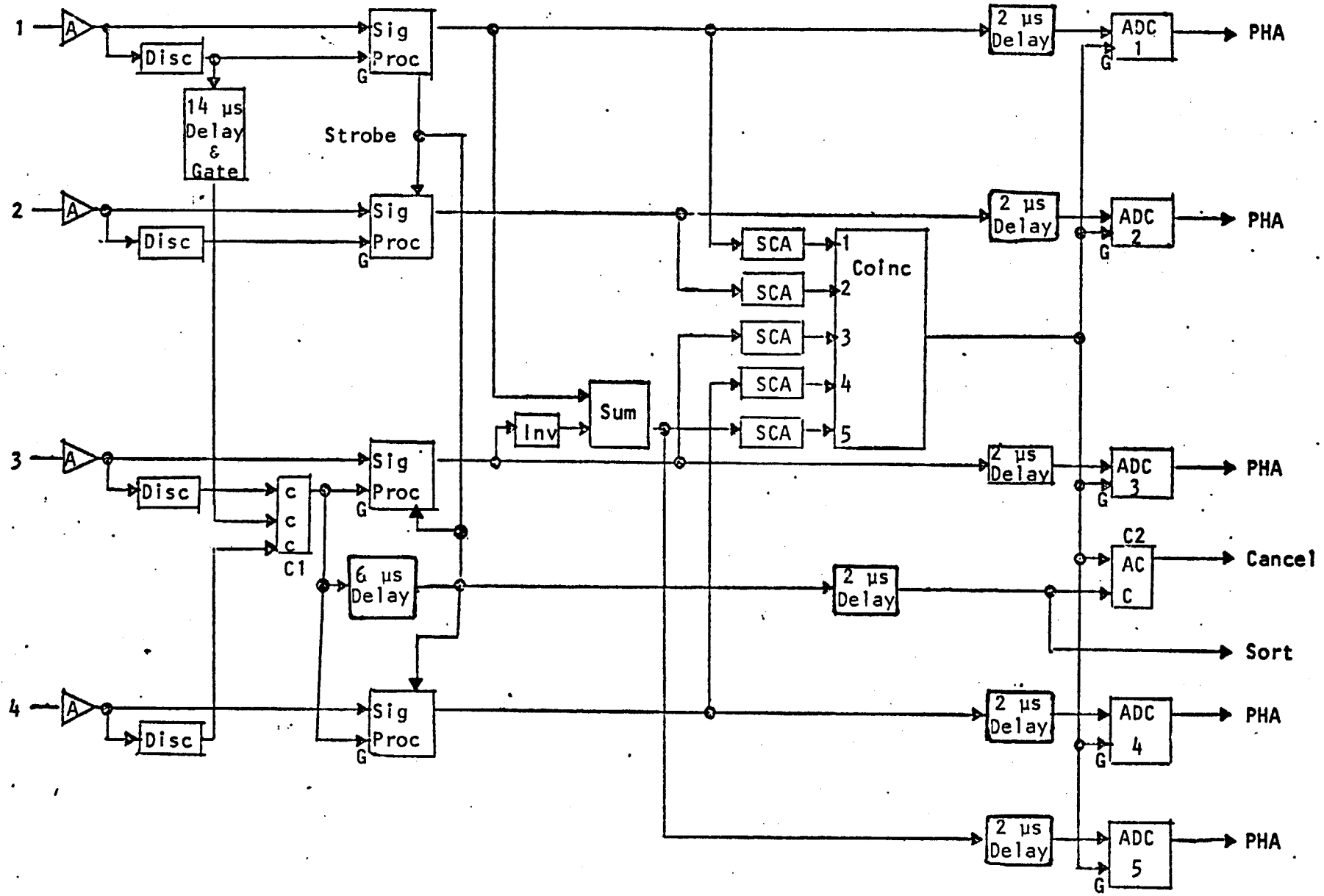
Figure 1

Analysis and sorting logic previously used on the dual beam sorter.

Four detectors (Det), preamplifiers (PA), and amplifiers (A) are used. The signals from the amplifiers go simultaneously to single channel analyzers (SCA) and to signal processors (Signal Proc). The signal processors are used for thresholding and for signal peak detection. The SCA's are preset so as to define sorting windows, and when a set of signals satisfies the window criteria and coincidence, a droplet charging pulse is generated. At the same time the signal processors are strobed to release the parameter values to the pulse height analyzers. This design is inadequate for the DBS as explained in the text.

FIGURE 2. ANALYSIS AND SORTING LOGIC PRESENTLY USED IN THE DUAL BEAM SORTER.

Signals from the amplifiers (A) are transmitted simultaneously to signal processors (Signal Proc) and discriminators (Disc). If the signal is above the preset threshold, the discriminator allows the signal processor to accept it as a valid signal. The 14 μsec delay and gate further guarantees that valid signals have the correct timing in order for signals to be released from the processors. The signals are then sent to single channel analyzers (SCA) for sorting decisions as in Fig. 1, and after a 2 μsec delay to the analog-to-digital converters (ADC) to be processed into a histogram.



into channel 3 and 4 after a 14 microsecond delay from a signal in channel 1. A signal in channel 3 or 4 that is caused by cross-talk from laser beam one will be simultaneous with the signal to channel 1 and will not be accepted by the signal processors for channel 3 or 4. Only signals from laser beam two (16 microseconds later) will be accepted as valid by the signal processors in channels 3 and 4. After a valid signal is received by the signal processor in channel 3 or 4, a strobe is activated to release simultaneously the valid signals in all the signal processors. The output from the signal processors goes both to analog-to-digital converters (ADC) to generate a histogram, and to single channel analyzers (SCA) which are used to establish sort windows for each parameter. A logic signal is generated by each SCA if the measured signal intensity falls within predetermined limits. When signal limits and coincidence requirements are met, the coincidence unit (Coinc.) generates an output pulse to allow acceptance of the signals by the ADC'S as well to generate a droplet charging pulse.

The inversion and sum circuitry and SCA-5 (Figure 2) are included so as to allow analysis and sorting based on the difference signal between channel 1 and channel 3. If the amplifiers are logarithmic (as is usually the case), this channel is equivalent to the logarithm of the ratio of the fluorescence intensities.

This signal handling system was designed and tested during the reporting period. It appears workable, and was utilized to generate two-and three-parameter data from cervical samples stained with Hoechst 33258 and Chromomycin A_3 or with SITS and Chromomycin A_3. When orthogonal light scatter is one of the measured parameters, some difficulties with timing and peak detecting functions still remain. Presumably the problems are related to the very complex peak shapes generated when the large epithelial cells pass through the relatively small laser slits in the DBS.

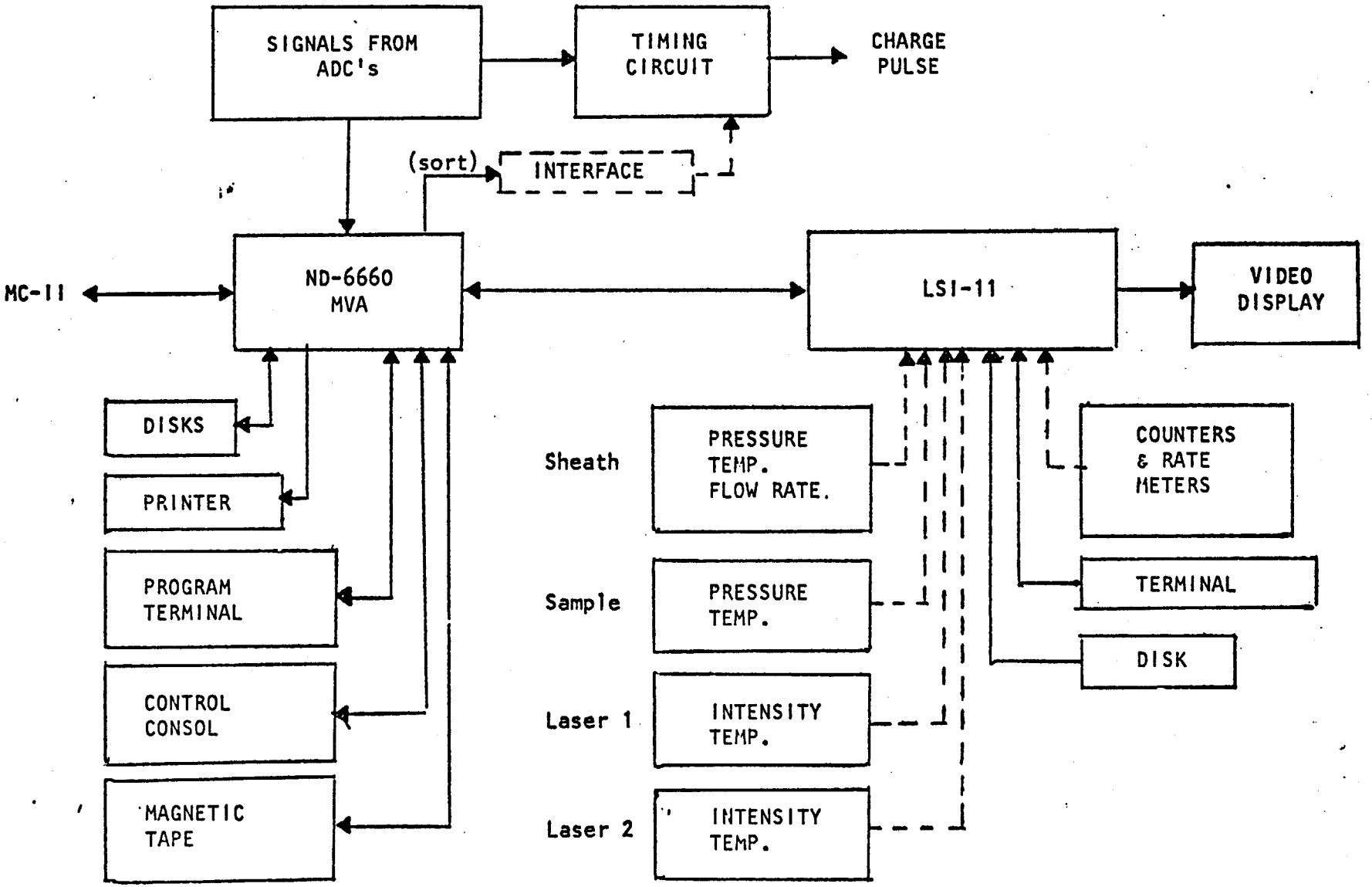
Routine sorting of cervical samples using this new signal processing circuitry has not been attempted. Instead, priority was assigned to accomplishing computer-controlled sorting.

Computer Controlled Sorting

The current status of computer control of our dual beam sorter (DBS) is illustrated in Figure 3. Solid lines indicate those connections that are completed, dashed lines those that are still under construction. The Nuclear Data Model ND-6660 computer-based multivariable analyzer has been interfaced to the Biomedical Sciences Division computer center (MC-II), to the LSI-11 micro computer used to monitor sorter performance and operation, and to the signal processing electronics shown in Figure 2. A magnetic tape unit was added to the analyzer to make it possible to store the large amounts of data obtained when measuring more than two variables for

FIGURE 3. BLOCK DIAGRAM OF COMPUTER CONTROLLED SORTING.

Computer controlled sorting uses three subsystems. The ND-6660 multi-variable analyzer (MVA) analyzes the detected signal intensities. It tests these signals against predetermined sorting criteria and generates sort commands to the timing circuit. The timing circuit generates a droplet charge at the time required for the cell to be at breakoff point. The LSI-11 monitors the sorter functions for precise control of the sorter. The solid lines indicate that link has been completed. The dotted lines indicate links that have not yet been completed.



each cell. The firmware necessary to enable control of sorting by the analyzer was received and installed. When the software is debugged, the computer will replace the SCA's and the PHA's in Fig. 2. We can then generate a sorting pulse based on any desired combination of measured variables, including non-rectangular windows, ratios, differences, sums, and complex combinations such as fluorescence anisotropy. The electronic circuits required to interface the computer generated sort signal to the timing and charge pulse generation circuits are under construction.

A video display has been added to the LSI-11. This display shows the running status of all sorter functions. A list of the functions that are monitored and displayed is shown in Figure 4. All of the information is updated at 1 second intervals. For record keeping, all of the information displayed can be transmitted to the ND-6660 and printed on its line printer. All pressure and temperature sensors have been obtained, along with the interface to the LSI-11, and will be installed during the summer. The counters and ratemeters have been installed and are now in operation.

Figure 4

LIST OF SORTER FUNCTIONS* DISPLAYED BY VIDEO FROM LSI 11

1. Number of cells analyzed
2. Number of cells sorted, right
3. Number of cells sorted, left
4. Count rates in each detector
5. Intensity of each laser beam
6. Temperature of each laser
7. Pressure in sheath and sample container
8. Temperature of sheath and sample
9. Sheath flow rate
10. PZT drive frequency
11. Number of drops sorted per deflection
12. Bookkeeping data (e.g., date, time of day, experimenter, wavelengths, etc.)

*The sorter functions are monitored continuously by the LSI-11 and the video output is redisplayed every second.

Development and Evaluation of New Markers

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Workscope

2. Continue development and evaluation of new markers for automation of clinical cytology screening utilizing spectroscopic and cytochemical techniques. The study of DNA and chromatin staining emphasizing DNA-DNA stain pairs will be carried out. Evaluation will also be conducted on the dual laser sorter.

4. Utilize the Lawrence Livermore Laboratory flow analysis/sorting systems to analyze clinical vaginal-cervical cytology specimens stained with mithramycin, chromomycin, PI/FITC and/or other fluorochromes as mutually agreed by the principal investigator and project officer. These specimens shall include but not be limited to normal, typical, mildly, moderately and severely dysplastic, carcinoma in situ and invasive carcinoma specimens.

5. Identify subpopulations in the specimens detailed in 4 above based on single and multiparameter analysis and sort these subpopulations.

6. The sorted subpopulations described in 5 above will then be morphologically characterized and restained for CYDAC analysis for quantitative comparison of individual cell types previously measured on CYDAC.

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Double Staining of Intracellular Chromatin

Development of double-DNA staining techniques for probing intracelluar chromatin structure in cervical cells has continued. Our previous approach has been twofold: i) to use the dual beam sorter and dual laser cytometer to analyze double stained cervical samples with three parameters, two based on DNA stains and one based on cell size; ii) to use the microfluorophotometer to identify individual cells morphologically and then measure the fluorescence from each of two DNA stains for each preidentified cell.

The second approach has indicated that for Hoechst-chromomycin labeled samples, the fluorescence ratio (F(ex360) /F (ex436))* is somewhat higher for cytomorphologically abnormal cells than for cytomorphologically normal cells (see e.g., Ninth Semi-Annual LLL Progress Report). Similar results were obtained for samples stained with chromomycin and propidium iodide and analyzed for the fluorescence ratio (F(ex546) /F (ex436)). Because of these encouraging results, the flow cytometric approach was of high priority.

Two-and three-parameter histograms have been obtained with Hoechst-chromomycin and chromomycin-propidium iodide stained cervical samples, and sorting of such samples has been performed, however, sorting capability and routine histogram analysis has been delayed frequently by hardware difficulties and by efforts to upgrade the dual beam sorter (For details on these changes see the earlier section on Dual Beam Sorter and Computer Controlled Sorting). Thus, real progress on this analysis awaits completion of the Dual Beam Computer Controlled Sorter.

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FOOTNOTE

The notation F(ex360) signifies fluorescence intensity following excitation at 360nm. The color of the fluorescence is not specified, so that the fraction of the signal due to energy transfer and that due to direct Hoechst fluorescence is not specified. These fractions can be measured, but are not needed to assess the potential of these parameters for diagnosis.

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FLOW ANALYSIS OF CHROMOMYCIN A_3-STAINED SPECIMENS

Preliminary tests have indicated that DNase pretreatment or elutriation pretreatment of cervical-vaginal samples may reduce the frequency of false alarms produced on flow cytometry using log (chromomycin A3 fluorescence) and log (orthogonal light scatter) as measured parameters.

To test whether DNase pretreatment improves cytometric results, each specimen was divided into halves. One half was prepared by our previous standard technique, and the other half was first exposed to 12.5 units per ml of DNase I for three minutes at room temperature, after which the cells were centrifuged and fixed with 70% ethanol as in our standard protocol.

The stained aliquots were analyzed by two-parameter flow cytometry, and the resultant histograms were compared for diagnostic power. An Atypia Index (A.I.) for each aliquot was calculated by taking the ratio of the number of signals in the Plain of Dysplasia to the number of signals due to all epithelial cells. The Plain of Dysplasia was defined in all histograms as Fl Channels 30-37 and Sc Channels 16-29, A second Atypia Index (A.M.) was also calculated by taking the ratio of the number of signals in the Plain of Dysplasia to the number of signals due to metaplastic and endocervical cells. The window defining the metaplastic and endocervical cell region was Fl Channels 21-27 and Sc Channels 15-23.

The results (Figures 5 and 6) indicate that, in general, DNase treatment increases the descrimination between abnormal specimens and normal specimens. However, the range of Atypia Indices for normal samples was so large the the descrimination between normal and abnormal specimens was not sufficient to significantly improve diagnostic power.

To further improve the diagnostic capability of flow cytometry, the four separate samples taken from each patient were not combined into a single tube, but instead were combined into two separate samples--one that contained the ectocervical scrape plus the endocervical sample (CE), and another that contained the vaginal wall scrape and the cervical-vaginal irrigation (VI). These separate samples for each specimen were then subjected to DNase pretreatment, ethanol fixation, and staining according to our standard protocol.

The stained aliquots were analyzed by two-parameter flow cytometry, and the resultant histograms were compared for their diagnostic power as described above for the DNase pretreatment. The results (Figure 7 and 8), show that, in general, the Atypia Indices of CE samples were higher than the Atypia Indices of VI samples.

The descrimination between normal and abnormal samples was best using A.I. of CE samples. Figure 9 shows a System Operating Characteristic Curve generated using those data. The best operating point on that curve results in 33% False Negatives (4/12) and 43% False Positives (9/21).

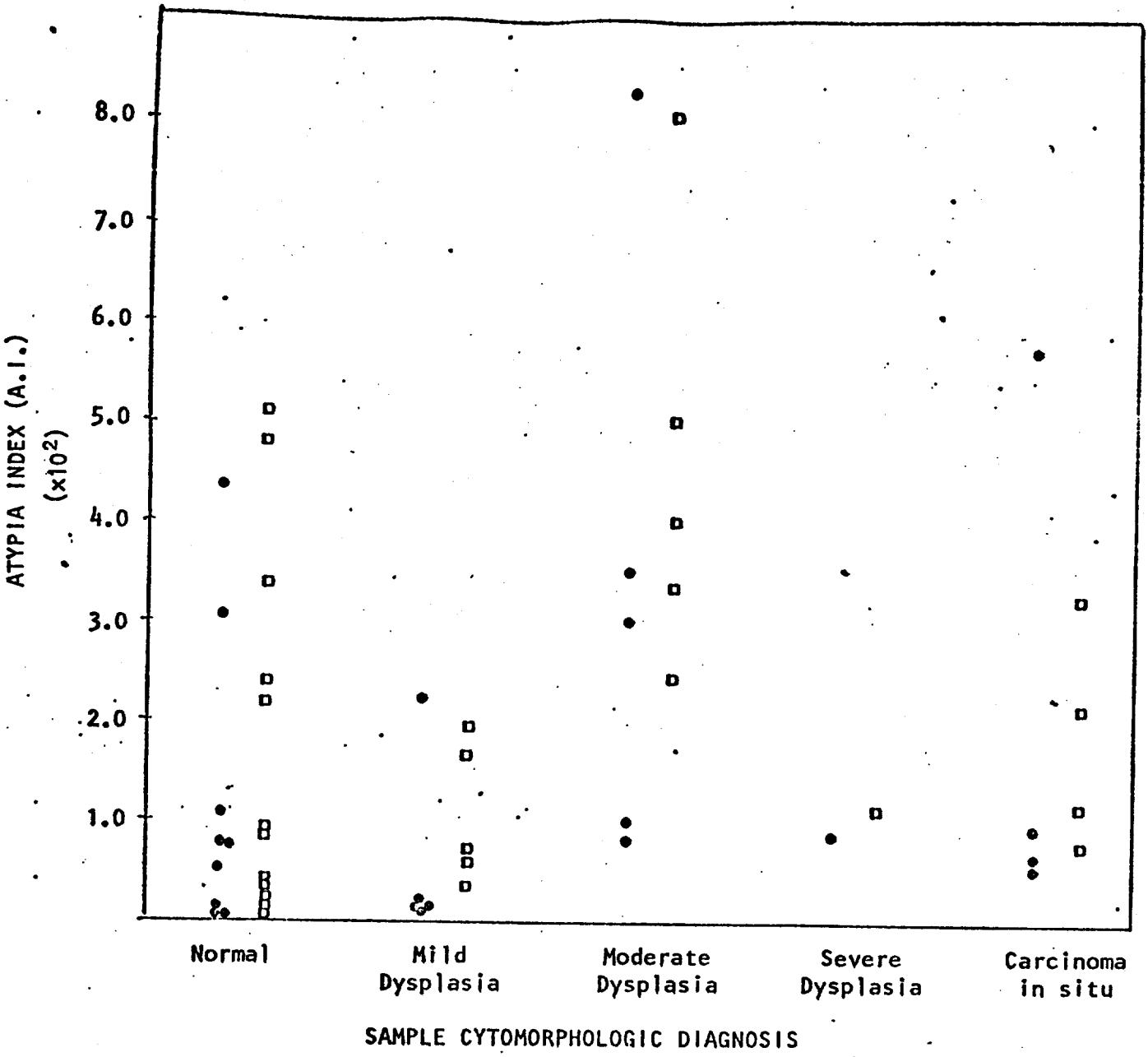

FIGURE 5. COMPARISON OF FLOW CYTOMETRIC ANALYSIS AND CYTOMORPHOLOGIC DIAGNOSIS OF GYNECOLOGIC SPECIMENS WITH AND WITHOUT DNase PRETREATMENT

Specimens were divided into two aliquots and either treated with DNase -□, or left untreated -●. After fixing and staining according to our standard protocol, histograms were measured by flow cytometry measuring log (chromomycin A_3 fluorescence) and log (orthogonal light scatter). The Atypia Index (A.I.) was calculated by taking the ratio of the number of signals in the Plain of Dysplasia (F1 channels 30-37, Sc channels 16-29) to the number of signals from all epithelial cells (F1 channels 21-64, Sc channels 16-64). An independent cytomorphologic diagnosis was performed on an aliquot of each sample. The A.I. is plotted for each sample relative to its cytomorphologic diagnosis.

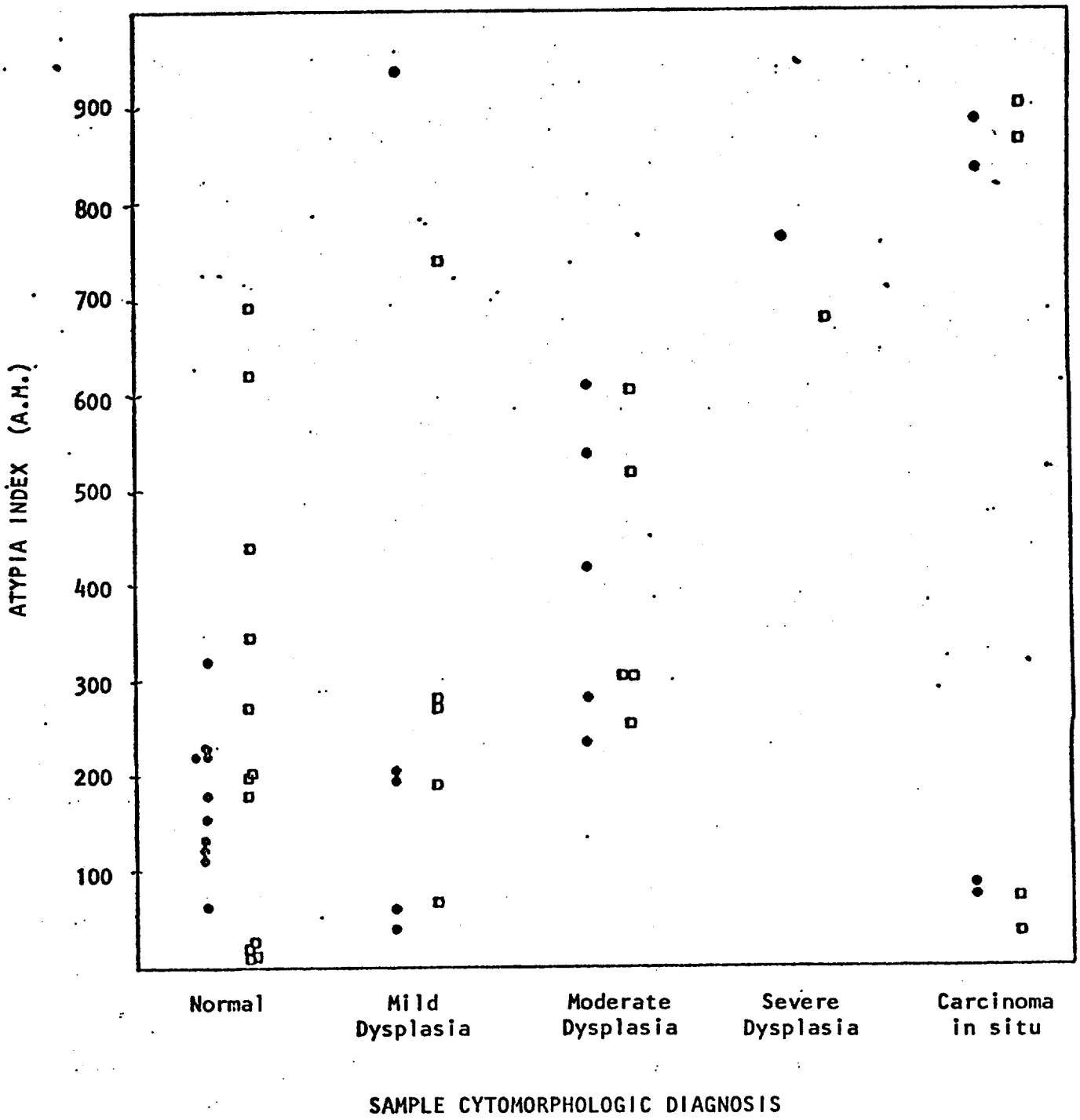

FIGURE 6. COMPARISON OF FLOW CYTOMETRIC ANALYSIS AND CYTOMORPHOLOGIC DIAGNOSIS OF GYNECOLOGIC SPECIMENS WITH AND WITHOUT DNase TREATMENT

Specimens and treatment are identical with those used for Figure 5. Atypia Index (A.M.) was calculated by taking the ratio of the number of signals in the Plain of Dysplasia (Fl. channels 30-37, Sc channels 16-29), to the number of signals in the region of metaplastic cells (Fl channels 21-27, Sc channels 15-23). The Atypia Index is plotted for each category of diagnosis with -□ or without -● DNase treatment.

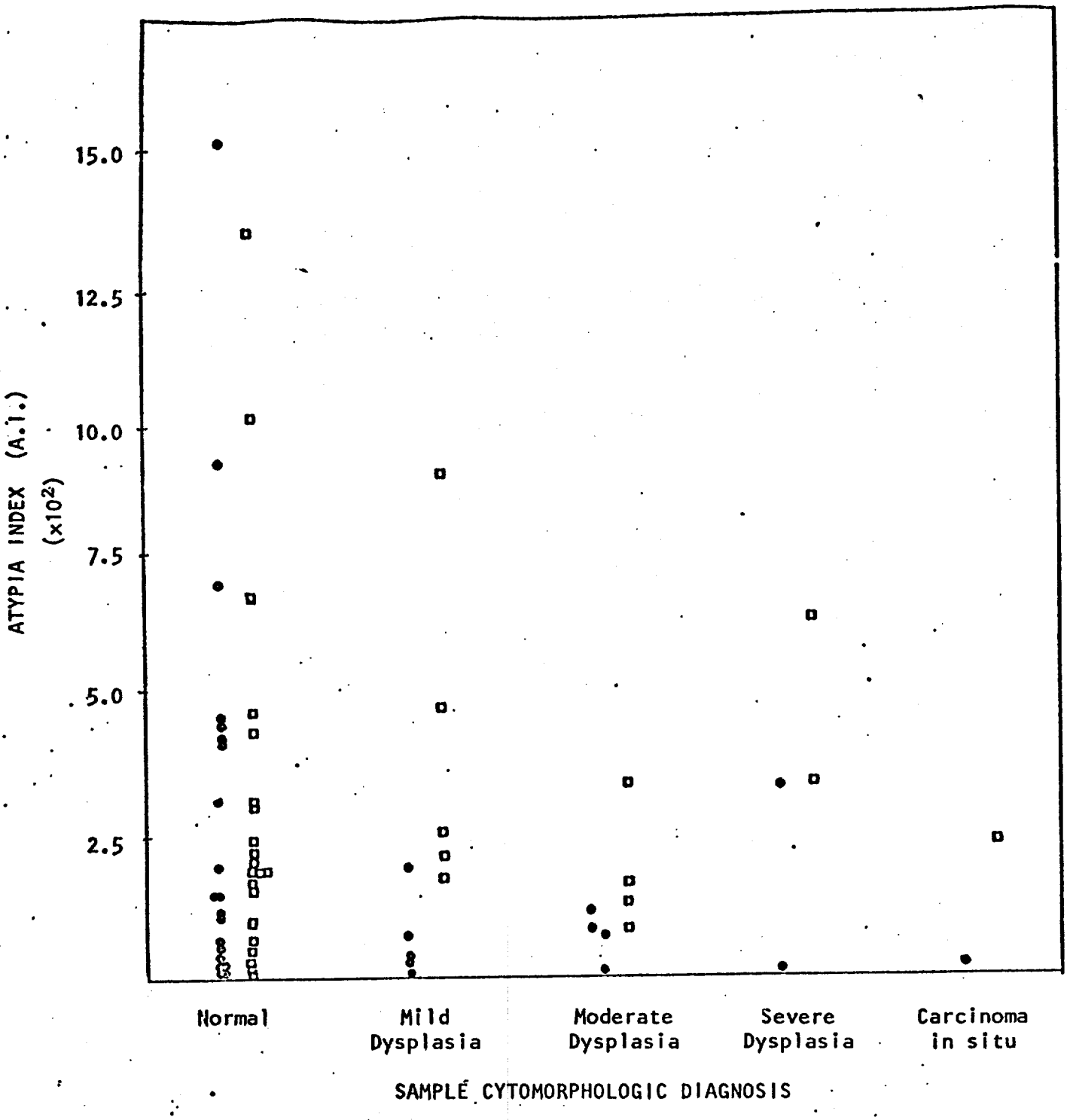

FIGURE 7. COMPARISON OF FLOW CYTOMETRIC ANALYSIS AND CYTOMORPHOLOGIC DIAGNOSIS OF GYNECOLOGIC SPECIMENS FROM THE CERVIX AND THE VAGINA

Samples were obtained from the ecto- and endocervix and from the vaginal wall. They were pooled separately as described in the text, treated with DNase, fixed, stained and analyzed by our standard protocol. The Atypia Index (A.I.) was determined as in Figure 5, for the vaginal specimen -● and the cervical specimen -□.

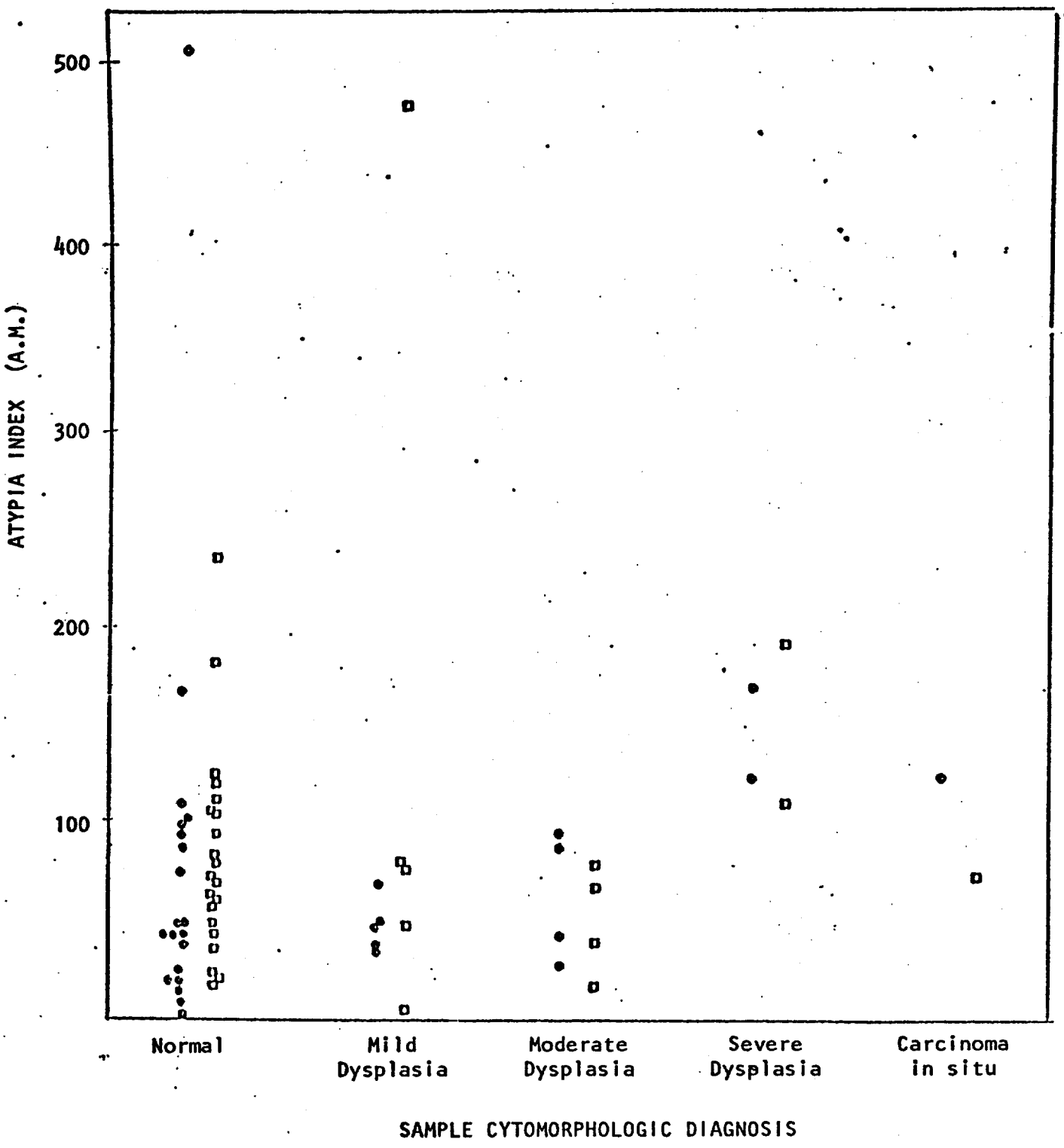

FIGURE 8. COMPARISON OF FLOW CYTOMETRIC ANALYSIS AND CYTOMORPHOLOGIC DIAGNOSIS OF GYNECOLOGIC SPECIMENS FROM THE CERVIX AND THE VAGINA

Samples were obtained from the endo and ectocervix and from the vaginal wall. They were pooled separately as described in the text, treated with DNase, fixed, stained, and analyzed according to our standard protocol. The Atypia Index (A.M.) was calculated as described for Figure 6, for the vaginal specimen - ●, and the cervical specimen - □.

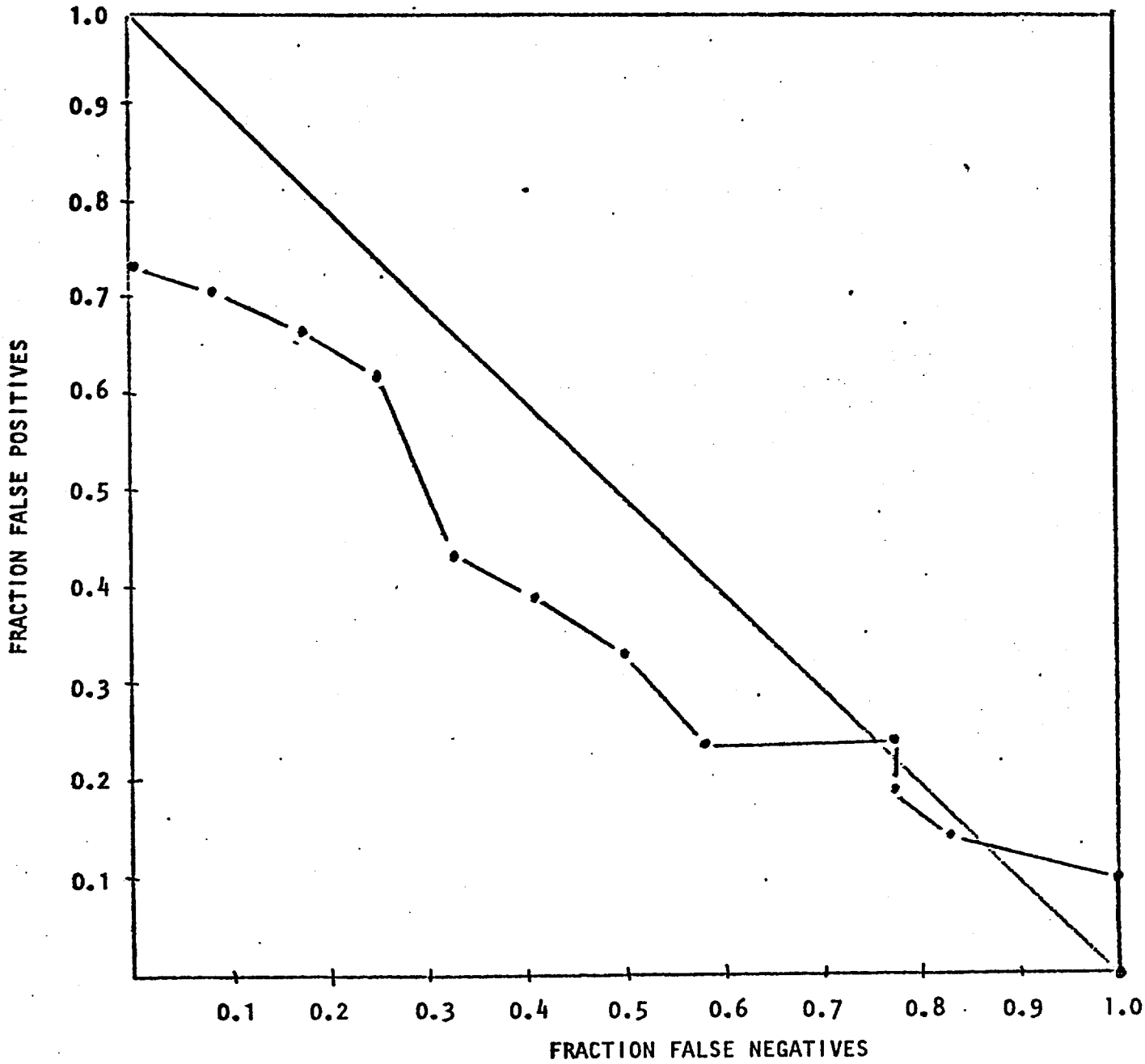

FIGURE 9. SYSTEM OPERATING CHARACTERISTIC CURVE FOR FLOW ANALYSIS OF CERVICAL SPECIMENS.

Samples were obtained, treated and analyzed as described for Figure 7. The flow cytometric data were compared with the cytomorphologic diagnosis by defining 11 different thresholds between positive and negative in the Atypia Index (A.I.). Each threshold generated a point in the plot of False Positives or False Negatives.

Again the main difficulty is the tremendous spread in the A.I. of normal samples.

We conclude that these two improvements in sampling and preparation are not sufficient to warrant their incorporation into a routine protocol for diagnostic prescreening. Other improvements in sample preparation will be tested if they appear promising.

Morphologic Cytology and Sample Preparation

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Workscope

7. Provide support for UCSF for specimen collection and preparation and evaluation according to the currently defined procedures.

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The UCSF Dysplasia Clinic continues to collect and supply specimens with cytomorphologic analysis on aliquots of the same specimens. Table I summarizes the cytomorphologic diagnoses of specimens obtained in this manner from patients in the dysplasia clinic during the present reporting period.

In addition we required a number of samples from the general population as more representative of normal. These specimens were obtained from paid volunteers who had no previous history of dysplasia or of herpes infection. Table II summarizes the cytomorphologic diagnoses of the specimens from these donors.

Also during the reporting period a test was performed in which samples from the vaginal wall, the endocervical canal, the cervical os, and a vaginal irrigation were not combined into one large specimen from each patient. Instead the endocervical and ectocervical sample (CE) were combined and the samples from the vaginal wall and the irrigation (VI) were combined separately. The cytomorphologic diagnosis of these samples is listed separately in Table III.

Note the very interesting result in Tables II and III that the "normal" donors showed dysplasia in five cases of the sixty-four obtained. These donors were informed of the results and are being followed carefully.

TABLE I

Summary of Cytomorphologic Diagnosis

Combined VICE Dysplasia Clinic Specimens from September 13, 1978 to June 19, 1979 (LLL 1832 to LLL 2031)

DIAGNOSES	NUMBER OF SPECIMENS
Benign	45
Benign + nonneoplastic changes (e.g. metaplasia)	15
Benign, few cells suggest mild dysplasia	12
Mild dysplasia	32
Mild to moderate dysplasia	7
Moderate dysplasia	14
Moderate to severe dysplasia	3
Severe dysplasia	6
Severe dysplasia to CIS	2
CIS	4
Invasive, squamous cell carcinoma	0
No diagnosis	1
TOTAL	141

TABLE II

Summary of Cytomorphologic Diagnosis

Combined VICE Specimens from Normal Volunteers from September 13, 1978 to June 19, 1979 (LLL 1832 to LLL 2120)

DIAGNOSES	NUMBER OF SPECIMENS
Benign	25
Benign + nonneoplastic changes (e.g. metaplasia)	11
Benign, few cells suggest mild dysplasia	0
Mild dysplasia	1
Mild to moderate dysplasia	0
Moderate dysplasia	0
Moderate to severe dysplasia	0
Severe dysplasia	1
Severe dysplasia to CIS	0
CIS	0
Invasive, squamous cell carcinoma	0
No diagnosis	0
TOTAL	38

TABLE III

Summary of Cytomorphologic Diagnosis

Separate VI and CE Specimens from Normal Volunteers and from

Dysplasia Clinic Patients from September 13, 1978 to June 19, 1979

(LLL 2032 to LLL 2120)

DIAGNOSES	NUMBER OF SPECIMENS			
	Dysplasia Clinic		Volunteers	
	VI	CE	VI	CE
Benign	8	6	11	11
Benign + nonneoplastic changes(e.g. metaplasia)	14	14	12	12
Benign, few cells suggest mild dysplasia	6	5	1	1
Mild dysplasia	18	15	2	2
Mild to moderate dysplasia	6	5	0	0
Moderate dysplasia	2	10	0	0
Moderate to severe dysplasia	2	1	0	0
Severe dysplasia	1	1	0	0
Severe dysplasia to CIS	2	2	0	0
CIS	2	2	0	0
Invasive, squamous cell carcinoma	0	0	0	0
No diagnosis	0	0	0	0
TOTALS	61	61	26	26

COLLARBORATIVE ACTIVITIES

a) Exchange Scientist Visit to Leiden, The Netherlands by Brian Mayall

Dr. Mayall began his exchange visit the first of May in the laboratory of Dr. J. Ploem at the University of Leiden. His intended subject of study is the application of the Leiden Texture Analyzing System (LEYTAS) to cell and chromosome morphology. A complete report will be submitted when he returns in September.

b) Hydrolytic Enzymatic Activities as Markers for Cervical Dysplasia

The enzymes gamma glutamyl transpeptidase, plasminogen activator, and heat resistant alkaline phosphatase continue to be studied at a minimal level. They continue to be promising markers; however lack of sufficient manpower prevents full scale investigation of possible probes for these markers.

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